THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 11, Issue of March 18, pp. 10834–10839, 2005
Printed in U.S.A.

Purified *Bacillus anthracis* Lethal Toxin Complex Formed *in Vitro* and during Infection Exhibits Functional and Biological Activity*

Received for publication, October 28, 2004, and in revised form, December 22, 2004
Published, JBC Papers in Press, January 11, 2005, DOI 10.1074/jbc.M412210200

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Anthrax protective antigen (PA, 83 kDa), a pore-forming protein, upon protease activation to 63 kDa (PA_{63}), translocates lethal factor (LF) and edema factor (EF) from endosomes into the cytosol of the cell. The relatively small size of the heptameric PA_{63} pore (~12 Å) raises questions as to how large molecules such as LF and EF can move through the pore. In addition, the reported high binding affinity between PA and EF/LF suggests that EF/LF may not dissociate but remain complexed with activated PA_{63}. In this study, we found that purified $(PA_{63})_7$-LF complex exhibited biological and functional activities similar to the free LF. Purified LF complexed with PA_{63} heptamer was able to cleave both a synthetic peptide substrate and endogenous mitogen-activated protein kinase kinase substrates and kill susceptible macrophage cells. Electrophysiological studies of the complex showed strong rectification of the ionic current at positive voltages, an effect similar to that observed if LF is added to the channels formed by heptameric PA_{63} pore. Complexes of $(PA_{63})_7$-LF found in the plasma of infected animals showed functional activity. Identifying active complex in the blood of infected animals has important implications for therapeutic design, especially those directed against PA and LF. Our studies suggest that the individual toxin components and the complex must be considered as critical targets for anthrax therapeutics.

Lethal and edema toxins play key pathogenesis roles as virulence factors produced by *Bacillus anthracis*, the etiologic agent of anthrax. The toxins show commonality in that both of their enzymatic components, lethal factor (LF,[1] 90 kDa) and edema factor (EF, 88 kDa), require protective antigen (PA) for translocation into host cells. LF, a Zn^{+2}-dependent metalloprotease, cleaves several members of the mitogen-activated protein kinase kinase (MAPKK) family (1–4), and in complex with PA, is responsible for the lethal action of anthrax toxin. EF is a calcium- and calmodulin-dependent adenylate cyclase that elevates intracellular levels of cyclic AMP, causing deregulation of cellular physiology and resulting in edema (5).

The proposed *in vitro* model for the binding, assembly, translocation, and subsequent killing of the target cells by anthrax lethal toxin involves a series of steps (6). PA binds to the ubiquitous cellular receptors tumor endothelial marker 8 (TEM8) (7) and capillary morphogenesis protein 2 (CMG2) (8). Proteolytic cleavage of PA by cell surface proteases such as furin (9, 10) generates a 63-kDa fragment that oligomerizes and forms a ring-shaped heptameric prepore. The LF and EF proteins bind to the prepore, and the whole complex then undergoes receptor-mediated endocytosis. The acidic environment in the endosomes causes PA to undergo a conformational change that promotes membrane insertion and formation of a transmembrane channel. In a recent study, this process of insertion was shown to occur in the early endosomes, whereas the delivery of the enzymatic moieties required the involvement of the late endosomes (11). LF in the cytosol cleaves MAPKK (3, 12, 13) and thereby inhibits one or more signaling pathways. Disruption of the signaling cascade by lethal toxin impairs the host immune response in macrophages (2–4, 14) and dendritic cells (15), thereby allowing the growth and infection of the bacterium. To date, the direct causal relation between inactivation of the MAPK signaling and lethal toxin-induced cytotoxicity remains unclear.

Under saturating conditions, the PA heptamer in solution binds to three molecules of the ligand (16). Prior studies by Singh *et al.* (17) showed that PA_{63} in either the monomeric or the oligomeric form can bind LF in a 1:1 ratio. Nevertheless, both the enzymatic moieties EF and LF bind the heptamerized PA_{63} competitively and with high affinity (K_d ~1 nM) (18). This interaction is not affected by low pH conditions (pH 5.2) (18). To date, there is no direct evidence showing that full-length LF dissociates from the PA_{63} heptamer complex either during or after translocation through the PA pore or that LF bound to the PA_{63} heptamer is capable of cleaving its substrates. In this study, we provide experimental evidence showing that LF bound to the PA_{63} heptamer is active and exhibits biological and biophysical properties similar to those of free LF.

* This work was supported by the Defense Advance Research Projects Agency (to J. W. E.), NIST ATP, SM3, NSF (NIRT Grant CTS-0304062 to J. J. K), Defense Threat Reduction Agency/JSTO-CBD, and USAMRIID project numbers 0242C012 (to S. B.) and 02-4-CC-010 (to B. P.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LF, lethal factor; EF, edema factor; PA, protective antigen; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; dnp, 2,4-dinitrophenyl; MCA, 4-(7-methoxycoumaryl)acetic acid.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
18 MAR 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Purified Bacillus anthracis lethal toxin complex formed in vitro and during infection exhibits functional and biological activity, Journal of Biological Chemistry 180:10834 - 10839	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Panchal, RG Halverson, K Ribot, W Lane, D Kenny, T Abshire, TG Ezzell, JW Hoover, TA	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	**RPP-05-100**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Anthrax protective antigen (PA, 83 kDa), a pore-forming protein upon protease activation to 63 kDa (PA63), translocates lethal factor (LF) and edema factor (EF) from endosomes into the cytosol of the cell. The relatively small size of the heptameric PA63 pore (~12 A) raises questions as to how large molecules like LF and EF can move through the pore. In addition, the reported high binding affinity between PA and EF/LF suggests that EF/LF may not dissociate but remain complexed with activated PA63. In this study, we found that purified (PA63)7:LF complex exhibited biological and functional activities similar to the free LF. Purified LF complexed with PA63 heptamer was able to cleave both a synthetic peptide substrate and endogenous mitogen-activated protein kinase kinase substrates and kill susceptible macrophage cells. Electrophysiological studies of the complex showed strong rectification of the ionic current at positive voltages, an effect similar to that observed if LF is added to the channels formed by heptameric PA63 pore. Complexes of (PA63)7:LF found in the plasma of infected animals showed functional activity. Identifying active complex in the blood of infected animals has important implications for therapeutic design, especially those directed against PA and LF. Our studies suggest that the individual toxin components and the complex must be considered as critical targets for anthrax therapeutics.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, lethal toxin, purification, function

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**6**	

MATERIALS AND METHODS

Purification of $(PA_{63})_7$-LF Complex—Purified PA_{83}, PA_{63}, and LF proteins (List Biological laboratories Inc.) from *B. anthracis* were used for these studies. Purified PA_{63} and LF proteins were mixed at either a 3:2 or 3:1 molar ratio of PA_{63} monomer:LF monomer and incubated for 30 min at room temperature. The resulting $(PA_{63})_7$-LF complex was purified over a Superose 6 size exclusion column (Amersham Biosciences), previously equilibrated with phosphate-buffered saline, pH 7.4. The total protein concentration of the complex fraction was determined by the Bradford protein assay reagent (Bio-Rad). The protein concentration of LF in the complex was determined by enzyme-linked immunosorbent assay. Briefly, 2-fold serially diluted LF protein or the complex was coated on microtiter plates (Nunc) for 16 h at 4 °C. The wells were washed four times with Tris-buffered saline-Tween-20 (0.1%) and incubated with anti-LF polyclonal antibody (1:500) for 2 h at room temperature. Wells were washed as above and incubated with peroxidase-conjugated goat anti-rabbit antibody at 1:2000 dilution for 2 h at room temperature. Wells were washed, and reactivity was developed with one-component 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) peroxidase substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). Color development was monitored at a wavelength of 405 nm. A calibration curve was established using the LF protein as a standard, and LF concentration in the complex was determined based on the standard curve.

LF Activity Assay—LF activity in the complex was determined using a fluorescence plate-based assay, as described previously (19). Briefly, a reaction mix containing 40 mM Hepes buffer, pH 7.2, 0.05% Tween 20, and different concentrations of free LF or complexed LF was added to each well. The reaction was initiated by adding 20 μM optimized peptide substrate (20) MCA-KKVYPYPME(dnp)K amide, and kinetic measurements were obtained every minute for 30 min using a fluorescence plate reader (Tecan, Safire). The peptide has an excitation peak at 324 nm and an emission peak at 395 nm.

Alternatively, LF activity was also measured after capture of the complex using PA antibody. The PA monoclonal antibody (PAI 3F10) was bound to Nunc Immuno Maxisorp surface (Nunc) plates for 2 h at room temperature and then blocked overnight with Tris-buffered saline-Tween-20 (0.1%) containing 6% nonfat dry milk at 4 °C. The plate was washed three times with Tris-buffered saline-Tween-20, and purified complex (either 200 ng/well or 20 ng/well of LF in complex form) was added to the antibody plate and incubated for 2 h at room temperature. After extensive washing, the reaction mixture containing 20 μM fluorogenic LF peptide substrate was added to the appropriate wells and incubated for 30 min. Cleavage of the peptide by bound LF was monitored by an increase in fluorescence intensity in a plate reader.

An HPLC-based LF activity assay was used to determine the kinetic constants (V_{max} and K_m values) of both LF in complex and free LF (19). Reaction mixture (30 μl total volume) containing 40 mM Hepes buffer, pH 7.2, 0.05% Tween 20, and seven different concentrations of peptide substrate ranging from 2 to 40 μM was incubated with free LF or LF in the complex (1 μg/ml) for 5 min at 30 °C. The reaction was stopped by adding 8 M guanidine hydrochloride in 0.3% trifluoroacetic acid. Substrate and products were separated on a Hi-Pore C18 column (Bio-Rad) using 0.1% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid/70% acetonitrile (solvent B). The column effluent was monitored at 365 nm, at which the substrate and C-terminal cleavage products had the strongest absorbance.

MAPKK Cleavage by Western Blot—J774A.1 macrophage cells ($\sim 1 \times 10^6$) seeded in a 6-well plate were incubated with either anthrax toxin (PA (88 ng/ml) and LF (16 ng/ml)) or purified complex (20 ng/ml or 200 ng/ml LF in complex form) for 4 h at 37 °C. Cells were harvested and lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1% Triton X-100, 10 mM NaF, 1 mM sodium orthovanadate, protease inhibitor mixture (Sigma), and phosphatase inhibitor mixture I and II (Sigma). The cell lysates were incubated for 30 min on ice and then centrifuged for 30 min at 14,000 rpm. Cell extracts (30 μg) were electrophoresed on SDS-PAGE and then subjected to Western blotting. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL).

Collection of Plasma from Infected Animals—Rabbits and guinea pigs that appeared moribund were anesthetized with a combination of ketamine, xylazine, and acepromazine under the guidance of staff veterinarians. Blood was drawn directly into tubes containing EDTA to inhibit proteases known to cleave PA_{83} to PA_{63}, mixed, centrifuged, and filtered through a 0.2-μm filter and stored at −70 °C. Plasma from the infected animals was then used to detect LF activity in the $(PA_{63})_7$-LF complex using either the antibody plate capture assay or electrophoresis, in a 4–15% native or SDS gradient gel for immunoblot analysis of the complex.

Antibodies—The antibodies used in this study include MEK1 NT (Upstate Biotechnology, Charlottesville, VA) and MEK2 NT, MEK3 C-19, MEK4 N terminus (Santa Cruz Biotechnology, Santa Cruz, CA). The PA and LF proteins in the complex were detected with rabbit anti-PA polyclonal antibody and a previously characterized LF monoclonal antibody (LFIII-5D2-1-1), respectively (21).

Cell-based Cytotoxicity Assay—J774A.1 cells were seeded at a density of 6×10^4 cells/well in a 96-well plate. The next day, cells were treated with 2-fold serially diluted anthrax toxin (PA (500 ng/ml) and LF (500 ng/ml)) or complex (500 ng/ml LF in complex). After a 4-h incubation with the toxin, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/ml) dye was added, and the cells were further incubated for 2 h. The reaction was stopped by adding an equal volume of lysis buffer (20% *N*,*N*-dimethylformamide and 20% SDS, pH 4.7). Plates were incubated overnight at 37 °C, and absorbance was read at 570 nm in a multiwell plate reader. Experiments were performed in duplicate and repeated three independent times for the toxin and the complex samples. The results are given as averages with standard deviation.

Electrophysiology—Channel recordings were carried out with planar lipid bilayer membranes as described previously (22). Briefly, solvent-free diphytanoyl phosphatidylcholine membranes were formed on a 50–100-μm-diameter hole in a thin Teflon partition. The partition separated two identical Teflon chambers that each contained ~2 ml of aqueous solution (0.1 M KCl, 5 mM MES, pH 6.6). Voltage was applied across the membrane via Ag-AgCl electrodes. The current was amplified using a patch clamp amplifier (Axon Instruments 200B), recorded with an analog to digital converter (DigiData 1322), and analyzed off-line. A negative transmembrane potential drove anions from the *cis* to the *trans* chamber. Details of this particular method were summarized elsewhere (23).

Channels were formed by adding small aliquots (~100 ng) of the purified $(PA_{63})_7$-LF complex to the aqueous electrolyte solution bathing one side of the membrane (called *cis*). The formation of individual channels was indicated by the stepwise changes in ionic current monitored at −50 mV applied potential. Data acquisition began after the current achieved a steady-state value. We applied brief (0.5-s) voltage pulses (typically +160 to −160 mV in 10-mV steps) across the membrane and averaged the first 100 ms of the signal to obtain the instantaneous current-voltage (*I-V*) relationship.

RESULTS

Purification and Characterization—To generate the $(PA_{63})_7$-LF complex, activated PA_{63} was incubated with LF either in 3:2 (Fig. 1*A*) or in 3:1 (Fig. 1*B*) molar ratio of PA_{63} monomer to LF monomer, to demonstrate the titration of free LF. The complex was isolated by size-exclusion chromatography. Peak fractions were collected and analyzed by native PAGE (Fig. 1*C*) and SDS-PAGE (Fig. 1*D*). As seen in Fig. 1*A*, a small amount of aggregated protein eluted first followed by the peak corresponding to the $(PA_{63})_7$-LF complex. Unbound or free LF eluted last. The protein peak corresponding to high molecular weight aggregate was not detectable by Coomassie Blue staining; however, some LF activity was detected in this fraction (data not shown), suggesting that this aggregated protein does contain some LF. Electrophoresis of PA_{63} (Fig. 1*C*, *lane 2*) on native gel showed a smear, probably due to complexes of different molecular sizes. However, binding of LF to PA_{63} resulted in a distinct $(PA_{63})_7$-LF complex band (Fig. 1*C*, *lanes 3* and *5*). SDS-PAGE of purified $(PA_{63})_7$-LF complex shows the presence of LF, PA_{63} monomer, and SDS-stable and heat-resistant PA_{63} heptamer (Fig. 1*D*, *lane 3*).

Alternatively, complex was also prepared and purified by mixing trypsin cleaved PA_{63} and LF proteins (data not shown). The peak fraction corresponding to the $(PA_{63})_7$-LF complex generated by either of the two methods was tested in different functional assays and displayed similar biological activity and biophysical properties.

Purified $(PA_{63})_7$-LF Complex Can Cleave Optimized Peptide Substrate—To test functional activity of LF in the complex, equal concentrations of either bound or free LF forms were

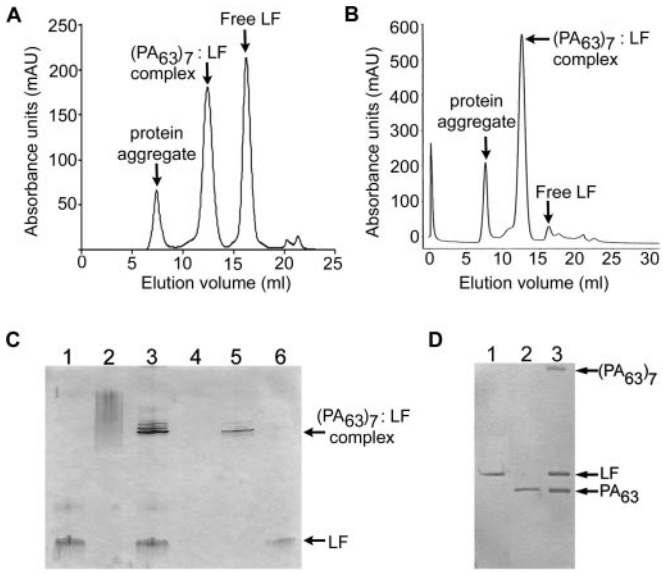

FIG. 1. **Purification and characterization of $(PA_{63})_7$-LF complex.** *A* and *B*, HPLC profiles of the preformed $(PA_{63})_7$-LF complex purified over a Superose 6 size exclusion chromatography column. *A*, a 3:2 molar ratio of PA_{63} monomer to LF monomer was used for the formation and isolation of the complex. *B*, to demonstrate titration of LF, a 3:1 molar ratio of PA_{63} monomer to LF monomer was used for the formation and isolation of the complex. *C*, Coomassie Blue staining of native PAGE. Samples were separated on a 4–15% gradient gel (PhastGel) and stained with Coomassie Blue. *Lane 1*, purified LF; *lane 2*, purified PA_{63}; *lane 3*, PA_{63}-LF mix before loading onto size exclusion column; *lane 4*, aggregated protein complex; *lane 5*, purified $(PA_{63})_7$-LF complex; *lane 6*, unbound or free LF. *D*, Coomassie Blue staining of SDS-PAGE gels. Samples were solubilized in SDS sample buffer, heated at 95 °C for 5 min, and electrophoresed on 4–12% gradient gel. *Lane 1*, purified LF; *lane 2*, purified PA_{63}; *lane 3*, purified $(PA_{63})_7$-LF complex.

incubated with an optimized peptide substrate that had a fluorogenic coumarin group at the N terminus and a 2,4-dinitrophenyl (dnp) quenching group at the C terminus (19). Kinetic measurements were made using a fluorescence plate reader at an excitation and emission maxima of 324 and 395 nm, respectively. As shown in Fig. 2*A*, LF in the complex form exhibits proteolytic activity, as it was able to cleave the peptide substrate and produce an increase in the fluorescence intensity.

To determine the rate of hydrolysis of the peptide substrate by LF in both the free and the bound forms, kinetics were studied by HPLC (19). Kinetic constants were obtained from plots of initial rates with seven different concentrations of the substrate. The V_{max} and K_m values for free LF were 1.8 ± 0.9 μmol/min/mg and 18.9 ± 0.14 μM, respectively. LF in the bound form had a V_{max} value of 2.45 ± 0.21 μmol/min/mg and a K_m of 25 ± 4.3 μM. Thus, LF showed a similar rate of enzyme-catalyzed hydrolysis of the peptide substrate whether in the free or in the bound form. A slightly higher K_m value for bound LF may indicate that the LF active site was not as accessible to the peptide substrate (with respect to free LF).

To address concerns about free LF contamination in the purified complex, we bound the complex to a PA monoclonal antibody coated onto a Maxisorp microtiter plate. After washing, the reaction mixture containing the optimized LF peptide substrate was added and incubated for 30 min. The increase in fluorescence intensity after peptide cleavage was measured using a fluorescence plate reader. As shown in Fig. 2*B*, LF activity was indeed observed when the complex was bound to the PA antibody plate, suggesting that LF in complex with PA_{63} heptamer was active. Furthermore, the activity of LF in the complex form was inhibited by NSC 12155, a previously reported small molecule LF inhibitor (19). Additionally, to show that free LF did not nonspecifically bind to the PA antibody, purified LF (10 μg/ml) was added to the PA antibody plate, and LF activity was measured (Fig. 2*B*). Subsequent analysis indicated no LF activity, suggesting that LF does not nonspecifically bind to the PA antibody.

Purified Complex Can Cleave MAPKK Substrates and Kill Susceptible Macrophage Cells—Anthrax LF is known to cleave several different isoforms of MAPKK both *in vitro* and in macrophage cells. To determine whether LF in the complex form could cleave endogenous MAPKK substrates, J774A.1 cells were treated with either the complex or the anthrax toxin (PA_{83} and LF), and cellular lysates were prepared and analyzed by Western blotting with antibodies against four different isoforms of MAPKK (Fig. 3*A*). The efficiency of the complex to cleave the four different isoforms of endogenous MAPKK was not significantly different from that of the toxin (PA_{83} and LF). A 2–10-fold excess of complexed LF was needed to obtain complete cleavage of endogenous MAPKK (compared with anthrax toxin), raising the possibility that not all the LF active sites in the complexed form were easily accessible to the endogenous substrate.

To test whether LF in the complex form was capable of killing susceptible macrophages, J774A.1 cells were treated with different concentrations of either the complex or the anthrax toxin and incubated for 4 h, and cell viability was measured by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 3*B*). Above 100 ng of LF, the killing efficiency between the complex and lethal toxin (mixed PA_{83} and LF) was not significantly different.

Purified Complex Forms Functional Channels in Planar Bilayer Membranes—Previous electrophysiological studies in planar bilayers demonstrated that PA_{63} heptamer at low pH (~ pH 6.5) forms cation-selective channels in lipid bilayers (24, 25) and in cell membranes (26). To study the biophysical properties of the $(PA_{63})_7$-LF complex, we first measured the ionic conductance of $(PA_{63})_7$ channels in both the absence and the presence of LF. Purified $(PA_{63})_7$ added to the electrolyte solution (0.1 M KCl, 5 mM MES, pH 6.6) spontaneously formed channels in bilayer membranes, as is shown in Fig. 4*A*, with each step in current (~4 pA at −50 mV applied potential) corresponding to the formation of a new channel. Interestingly, under similar conditions, adding purified $(PA_{63})_7$-LF complex also formed channels (Fig. 4*B*). However, the characteristic single channel current was ~1.8 pA (V = −50 mV). Thus, even with LF bound to the heptameric PA_{63}, it is still possible to form channels.

To mitigate the slow intrinsic gating of the $(PA_{63})_7$ channel (data not shown), we measured the instantaneous current-voltage relationship of membranes containing many channels of either $(PA_{63})_7$ (Fig. 4*C* without LF; Fig. 4*D* with LF in the aqueous phase) or $(PA_{63})_7$-LF complex (Fig. 4*E*) over a wide range of applied potential (*e.g.* −160 mV < *V* < 160 mV). In the absence of LF, $(PA_{63})_7$ channels exhibit current-voltage relationship that is only slightly nonlinear. In contrast, when ~1 nM LF was added to the *cis* subphase, the $(PA_{63})_7$ channels became extremely rectifying. Specifically, the conductance for positive potentials was much less than that for negative potentials (compare Fig. 4, *C* and *D*). Channels formed by the purified $(PA_{63})_7$-LF complex also strongly rectified (Fig. 4*E*) in a manner that is virtually identical to that of $(PA_{63})_7$ with LF added to the subphase (Fig. 4*D*). The averaged instantaneous *I-V* relationships for these particular experiments are shown in Fig. 4*F*. The results demonstrate that purified $(PA_{63})_7$-LF preformed complex formed ion-conducting channels that are asymmetrically blocked by LF in planar bilayer membranes.

Biologically Active Complex Detected in Plasma of Infected Animals—Our studies suggest that *in vitro* generated and pu-

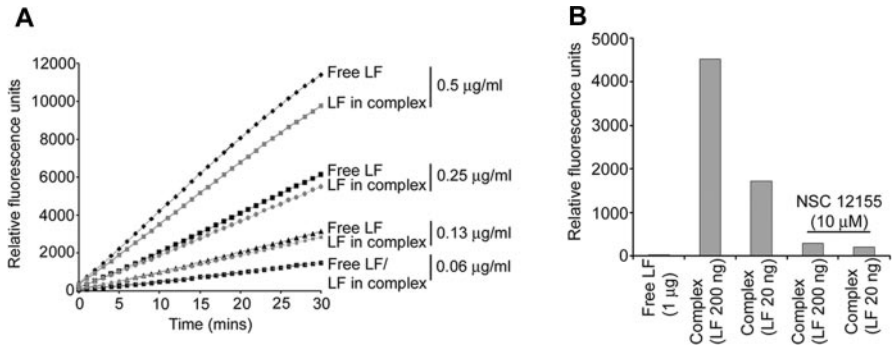

FIG. 2. **LF complexed with PA_{63} heptamer can cleave synthetic peptide substrate.** *A*, representative data of fluorescent plate reader assay showing that LF in the complex form exhibits similar activity to the free LF. Equal concentrations of either free LF or LF in complex form were incubated with 20 μM optimized peptide substrate (19), and kinetic measurements were obtained every min for 30 min at excitation and emission maxima of 325 and 394 nm, respectively. *B*, LF activity was observed when the complex was bound to the PA antibody plate. PA monoclonal antibody (PAI3F10) was bound to a MaxiSorp plate, blocked overnight, washed, and then incubated with the purified complex (either 200 ng/well or 20 ng/well of LF in complex form). After extensive washing, a reaction mixture containing 20 μM fluorogenic LF peptide substrate was added to the appropriate wells and incubated for 30 min. Cleavage of the peptide by bound LF was monitored by an increase in fluorescence intensity in a plate reader. As control, free LF (10 μg/ml) did not show any nonspecific binding to the PA monoclonal antibody. LF activity in the bound form could be inhibited by a previously identified small molecule LF inhibitor NSC 12155 (10 μM) (19).

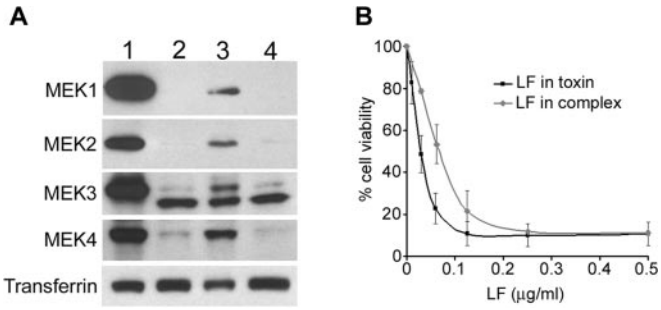

FIG. 3. **Purified $(PA_{63})_7$-LF complex exhibits biological activity.** *A*, proteolytic cleavage of endogenous MAPKK by LF in the complex. J774A.1 macrophages were either not treated (*lane 1*) or treated with anthrax toxin (88 ng/ml PA_{83} and 16 ng/ml LF) (*lane 2*), LF in complex (20 ng/ml LF in complex) (*lane 3*), or LF in complex at a different strength (200 ng/ml LF in complex) (*lane 4*) and incubated for 4 h at 37 °C. Cell lysates were prepared and analyzed by immunoblotting with antibodies specific to four different isoforms of MAPKK. A housekeeping transferrin protein was used to show uniform loading of the lysates. *B*, LF in the complex kills macrophages. J774A.1 cells were incubated for 4 h with 2-fold serial dilutions of anthrax toxin (starting with 0.5 μg/ml PA_{83} and 0.5 μg/ml LF) or complex (starting with 0.5 μg/ml LF in complex). Cell viability was then measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

rified $(PA_{63})_7$-LF complex is functionally and biologically active, but does such an active complex exist in an infected animal? Earlier studies by Ezzell and Abshire (27) showed the presence of a high molecular weight complex in the serum of anthrax-infected guinea pigs. PA was found to exist primarily as a 63-kDa protein in complex with LF, whereas no PA_{83} protein was detected in the infected blood. To identify a functionally active complex in the blood of infected animals, rabbits (n = 3) and guinea pigs (n = 2) were infected by aerosol challenge with a fully virulent *B. anthracis* strain. Just before death, the animals were anesthetized, and blood was collected in tubes containing EDTA. Plasma was isolated and added to the PA antibody plate, washed to remove nonbinding proteins, and then incubated in the presence of fluorogenic peptide substrate. Representative data from different infected animal species are shown in Fig. 5*A*. LF activity was detected in this assay, suggesting that there is a $(PA_{63})_7$-LF complex in the plasma of infected animals and that LF in the complex form is catalytically active. Incubation of fluorogenic peptide substrate with normal serum showed no increase in LF substrate cleavage as measured by fluorescence intensity, suggesting that cleavage of the peptide was not caused by serum proteases.

To visualize the banding pattern of the *in vivo* complex, infected plasma samples were subjected to native PAGE and analyzed by Western blotting using PA (Fig. 5*B*) and LF (Fig. 5*C*) antibodies. Infected plasma from three different rabbits (Fig. 5, *B* and *C, lanes 3–5*) and two different guinea pigs (Fig. 5, *B* and *C, lanes 6* and *7*) showed high molecular weight complexes of different sizes that were greater in size than the *in vitro* generated $(PA_{63})_7$-LF complex (Fig. 5, *B* and *C*, *lane 1*). The complex in all the three rabbits appears as a smear (Fig. 5, *B* and *C*, *lanes 3–5*). The complexes in the infected animals may have interacted and bound to blood-borne proteins, thereby affecting their mobility on native PAGE. Immunoblot with LF antibody showed some free LF in two of the infected rabbit plasma (Fig. 5*C*, *lanes 3* and *4*). No free PA_{83} was detected in the infected samples, as analyzed by Western blotting of both native PAGE (Fig. 5*B*) and SDS-PAGE (Fig. 5*D*, *top panel*). Control serum from untreated guinea pig (Fig. 5, *B* and *C, lane 2*) did not react with the PA or LF antibody. Plasma from representative infected animals, when subjected to SDS-PAGE and analyzed by immunoblotting with the PA antibody, showed PA_{63} monomer and SDS-resistant and heat-stable PA_{63} oligomer but no PA_{83} (Fig. 5*D*, *top panel*). The 90-kDa LF protein was detected with LF antibody (Fig. 5*D*, *bottom panel*) in the infected samples.

DISCUSSION

The widely accepted *in vitro* cell intoxication model explains how PA and LF might act in concert to disable the target. However, biochemical and cellular studies by different groups have poised several interesting questions. First, the reported high affinity interaction between LF and PA_{63} heptamer (18) suggests that LF may not dissociate from the PA_{63} heptamer, even under low pH (pH 5.2). Second, the relatively small size of the PA pore (inner diameter ~12–17 Å), estimated by single channel recordings (24, 28, 29) and from molecular modeling of the PA pore (30), suggest that the energy barrier to LF and EF translocation through the channel lumen may be formidable. However, pH-induced translocation studies in either Chinese hamster ovary or L6 cells revealed efficient translocation of a truncated LF fragment (LF_N, ~30 kDa) that contained the N-terminal PA binding domain (31). Their studies suggest that partial unfolding of the protein molecule is required during its passage through the PA channel (31). Electrophysiological measurements of PA_{63} channel blockade by EF_N (28) and LF_N (32) have also led to the hypothesis that EF and LF are translocated through the PA channels at pH 5.5. To date, there are

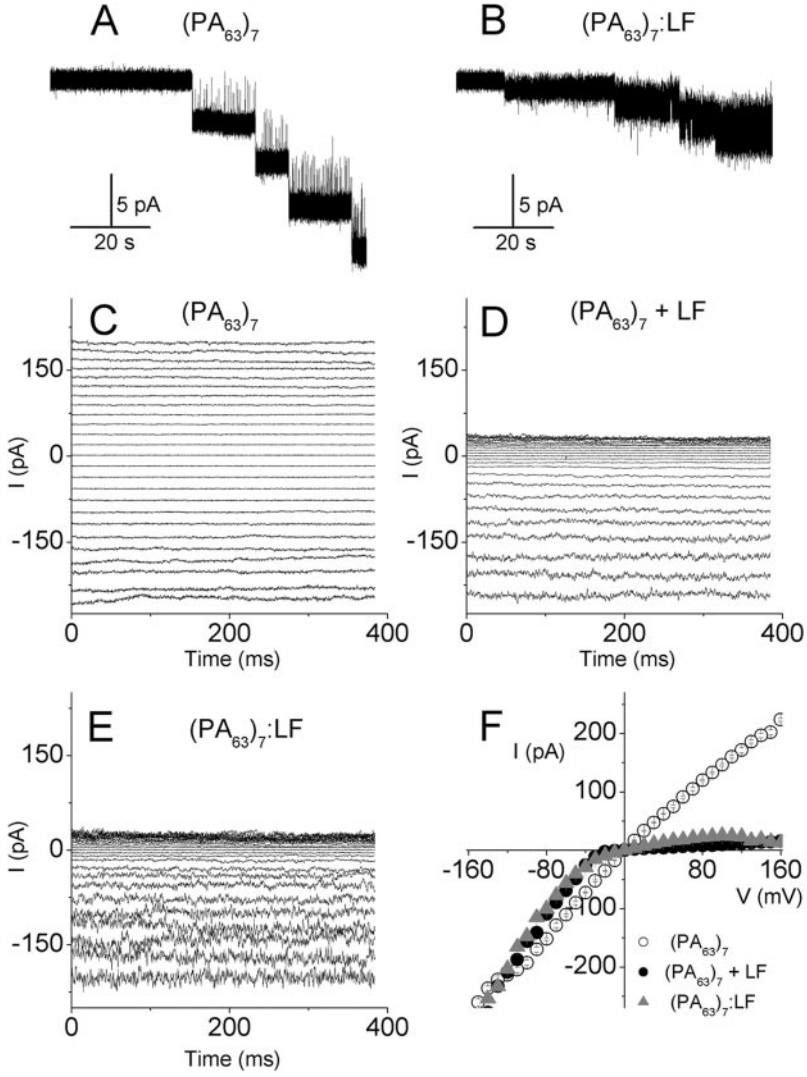

FIG. 4. **$(PA_{63})_7$ and purified $(PA_{63})_7$-LF complex form functional channels in planar bilayer membranes.** The discrete steps in ionic current in *A* and *B* correspond to the spontaneous formation of single channels of $(PA_{63})_7$ and purified $(PA_{63})_7$-LF complex, respectively. The applied potential was −50 mV. The representative data of instantaneous current-voltage (*I-V*) relationship for $(PA_{63})_7$ in the absence (*C*) and presence (*D*) of ~1 nM LF demonstrate that LF added to the *cis* compartment converted the $(PA_{63})_7$ *I-V* relationship from a slightly nonlinear to extremely rectifying. *E*, similar experiments show that channels formed from purified $(PA_{63})_7$-LF complex (< 200 ng of LF in the complex) also exhibited a highly rectifying *I-V* relationship. *F*, a plot of the *I-V* relationship of $(PA_{63})_7$, $(PA_{63})_7$ + 1 nM LF, and the purified $(PA_{63})_7$-LF complex shows that at positive applied potentials, LF blocked the pore, whether it was initially bound to the channel as a complex or added subsequently to the subphase bathing $(PA_{63})_7$ channels. The aqueous solutions on both sides of the membrane contained 0.1 M KCl, 5 mM MES at pH 6.6.

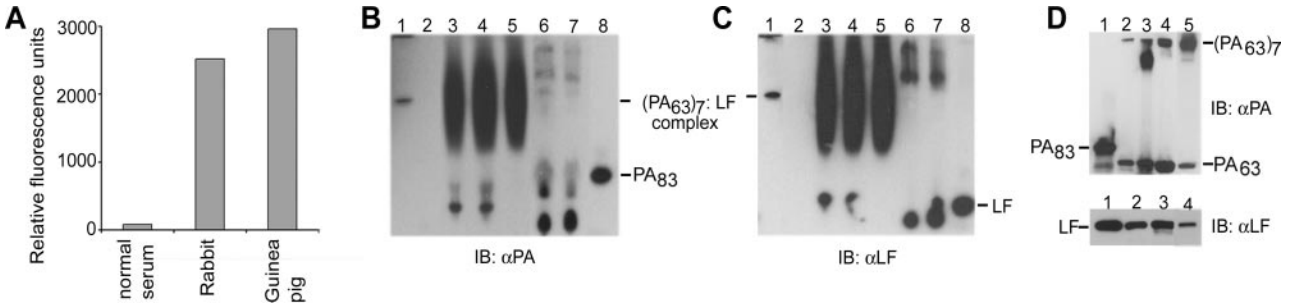

FIG. 5. **Detection of $(PA_{63})_7$-LF complex in plasma of infected animals.** *A*, representative data showing LF activity in $(PA_{63})_7$-LF complex from the plasma of infected animals. Plasma (50 μl) from infected animals or normal serum was bound to the PA antibody plate, washed to remove any nonspecific proteins, and then incubated with 20 μM fluorogenic peptide substrate for 30 min at room temperature. Cleavage of the peptide by LF in complex form was monitored by an increase in fluorescence intensity at an excitation and emission maxima of 325 and 394 nm, respectively. LF activity was detected, suggesting that $(PA_{63})_7$-LF complex in the plasma of infected animals is functionally active. *B* and *C*, high molecular weight $(PA_{63})_7$-LF complex detected in the plasma of different infected animals. *In vitro* generated and purified $(PA_{63})_7$-LF complex (*lane 1*), control untreated guinea pig serum (*lane 2*), plasma from three different infected rabbits (*lanes 3–5*), plasma from two different infected guinea pigs (*lanes 6–7*), and purified PA_{83} or LF protein (*lane 8*) were subjected to native PAGE, transferred onto nitrocellulose membranes, and then immunoblotted (*IB*) with either PA (*B*) or LF (*C*) antibody. *D*, PA_{83} was not detected in the plasma of infected animal. Representative data of samples subjected to SDS-PAGE and immunoblotted with anti-PA (*top panel*) and anti-LF (*bottom panel*) antibodies are shown. *Top panel*, PA_{83} (*lane 1*), PA_{63} (*lane 2*), *in vitro* purified complex (*lane 3*), plasma from infected guinea pig (*lane 4*) and plasma from infected rabbit (*lane 5*). *Bottom panel*, LF (*lane 1*), *in vitro* purified complex (*lane 2*), plasma from infected guinea pig (*lane 3*), and plasma from infected rabbit (*lane 4*).

no reported studies verifying the physical translocation of physiologically relevant full-length LF or EF molecules through the PA pore. The small size of the PA pore suggests that in order for the translocation process to occur, either EF and/or LF must be completely unfolded, or the PA pore and the lipid bilayer must undergo major conformational changes or a phase transition (33). To begin to address some of these questions, we purified $(PA_{63})_7$-LF complex and tested the biological activity of LF in the complex form.

In this study, we have clearly shown that purified LF com-

plexed with PA_{63} heptamer exhibits biological and functional activity. LF in the complex form cleaved both the synthetic peptide and the endogenous MAPKK substrates and killed susceptible macrophages. Moreover, there was no significant difference in the efficiency of cleavage and killing of susceptible cells by LF in complex or in the free LF form. These results suggest that LF does not have to dissociate from PA to exhibit biological activity and indeed can impart equivalent activity while still bound to PA. However, there is no direct evidence to support the presence of intact complex inside the cells.

In planar bilayer membranes, $(PA_{63})_7$ forms ionic channels, and LF converts the channels to extreme rectifiers. We found that $(PA_{63})_7$-LF complex also formed channels and that its conducting properties were virtually identical to those of $(PA_{63})_7$ channels in the presence of LF in the aqueous phase. The ionic channel recordings (Fig. 4, *A* and *B*) show that at low applied potential (−50 mV), the conductance of $(PA_{63})_7$ channels was approximately twice that of channels formed by $(PA_{63})_7$-LF complex (Fig. 4*F*). Thus, these results suggest that the channels formed by the purified complex were partially blocked by LF at small negative applied potentials, as were $(PA_{63})_7$ channels with LF in the subphase (Fig. 4*F*, $-80 \text{ mV} < V < 0$). Similar observations were made by Zhao *et al.* (34), who used a cell-based approach. Under acidic pH conditions (pH 5.5), LF caused a partial blockade of PA_{63} channels on the plasma membrane of Chinese hampster ovary-K1 cells (34). Thus, our data suggest that the *in vitro* preformed complex has functional properties similar to the complex formed by adding LF to PA_{63} heptamer in lipid bilayers or in cell-based studies, but it does not directly address the issue of LF translocation.

Detecting $(PA_{63})_7$-LF complex, but not PA_{83}, in the blood of infected animals suggests that the PA_{83} protein released by *B. anthracis* was efficiently activated by serum proteases. This is consistent with the studies by Ezzell and Abshire (27), which showed that only PA_{63} was detected in the blood of infected animals and appeared to be complexed with LF (and presumably with EF as well). In their studies, they found no free PA_{83} and proposed that the lethal/edema toxin are preformed in the blood at some point between the release of toxin components through the membrane of the bacillus and before binding to the host cell. Cleavage of the PA_{83} protein is catalyzed by a calcium-dependent, heat-labile protective antigen protease found in the serum of a wide range of animal species (27). EDTA inhibits this protease activity. As blood drawn from infected animals was collected in the presence of EDTA, cleavage of PA_{83} by proteases activated by the clotting cascade can be ruled out. An interesting observation in this study was the detection of functionally active $(PA_{63})_7$-LF complex in the blood of infected animals. Electrophysiological studies of purified $(PA_{63})_7$-LF complex from the plasma of infected guinea pigs strongly rectified the ionic current similarly to that observed with *in vitro* purified complex (data not shown). Although the complex in the blood could be detected only during the septicemia or the late stage of infection, data[2] suggest that the PA_{83} within *B. anthracis* is cleaved to PA_{63} on the bacterium surface by serum proteases. The PA_{63} appears to resist release from the bacteria, and the mechanism by which the predicted $(PA_{63})_7$-LF complex is released in the blood of infected animals has yet to be elucidated.

These findings raise questions about the role of the complex in anthrax pathogenesis. During the early stages of infection, sublethal dose of lethal toxin protects the bacteria by inactivating the immune system through MEK cleavage, thus preventing cytokine responses (4, 35, 36) and impairing adaptive immunity of dendritic cells (15). Whether the preformed complex has a similar role during the early infection process remains to be determined. In the late stage of infection, when a critical bacterial load or "point of no return" stage is reached, antibiotics kill the bacteria, but the animal still dies (37); therefore, design of adjunct anti-toxin therapeutics should consider the possibility that preformed complex is circulating in the blood of the terminal animals. If indeed the complex is formed in the early stages of infection, on the surface of the bacteria, then the timing of PA-directed therapeutics could be crucial. The individual toxin components and the complex must be considered as critical targets for anthrax therapeutics.

[2] J. W. Ezzell, Jr. and T. G. Abshire, personal communication.

Acknowledgments—We thank Rosemary Cushman for help in purifying the complex, Drs. James Schmidt and Martin Misakian for the useful comments, and Drs. Elizabeth Leffel and Brett Purcell for providing the infected plasma.

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